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Metalloproteases involved in the Temozolomide (TMZ) resistance of U87-MG glioma cells

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III. Abbreviations

ADAM	A Disintegrin and Metalloprotease
α 2-MG	α2-macroglobulin
AD	Alzheimer's disease
AIC	5-aminoimidazole-4-carboxamide
ATCC	American Type Culture Collection
APS	Ammonium persulfate
A β	Amyloid-β
BB-94	Batimastat
BSA	Bovine serum albumin
BCA	Bicinchoninic acid
°C	Celsius
cDNA	Complementary deoxyribonucleic acid
CD	Cytoplasmic domain
CO2	Carbon dioxide
CNS	Central nervous system
CTF	Carboxyl-terminal fragment
CNUs	Chloroethylnitrosoureas
DIS	Disintegrin
DDR	DNA damage response
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's modified eagle medium
dNTP	Deoxyribose nucleotide triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylendinitrilo-N,N,N',N',-tetra-acetate
ECM	Extracellular Matrix
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinases

FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
GBM	Glioblastoma multiforme
HRP	Horseradish peroxidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICDs	Intracellular domains
kDa	Kilo dalton
L1CAM	Cell adhesion molecule L1
MAPK	Mitogen-activated protein kinases
MPs	Metalloproteases
min	Minute
ml	Milliliter
MMPs	Matrix Metalloproteases
mRNA	Messenger RNA
MGMT	O^6 –methylguanine-DNA methyltransferase
MTT	dimethylthiazol-diphenyl tetrazolium bromide
MTIC	5-3-(methyl)-1-(triazen-1-yl)imidazole-4-carboxamide
NaCl	Sodium chloride
NAD	Nicotinamide Adenine Dincleotide
NCAM L1	Neural cell adhesion molecule L1
NTF	N-terminal fragment
PARs	Protease-activated receptors
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
PSEN	Presenilin
RIP	Intramembrane proteolysis
RT-PCR	Reverse transcription-PCR
RNA	Ribonucleic acid
ROS	reactive oxygen species

rpm	Rotations per minute
S2P	Site-2 protease
SCC	Squamous cell carcinomas
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SHH	Sonic Hedgehog
siRNA	Small interfering RNA
SPPs	Signal peptide peptidases
TEMED	N',N',N',N'-Tetra methyl diamine
TGF- β	Transforming Growth Factor-ß
Tris	Tris-(hydroxy methyl)-amino methane
TIMPs	Tissue inhibitors of metalloprotease
TM	Trans-membrane
TMA	Temozolomide acid
TMZ	Temozolomide
VEGFR-1	Vascular endothelial growth factor receptor-1

1 Introduction

1.1 Glioma as the most common malignant tumour occurring in the central nervous system

Glioma is the most frequent and malignant primary intracranial tumour. According to the World Health Organization (WHO)'s classification, glioma is divided into I-IV grades. I and II grades tumours are relatively benign, while III-IV grades tumours belong to most malignant ones, in particular the type of Glioblastoma multiforme (GBM). Even with multimodal treatment encompassing surgical resection, chemotherapy and radiotherapy, the mean survival of patients with GBM is less than 15 months after diagnosis (Louis *et al.*, 2007; Nieder *et al.*, 2006; Wong *et al.*, 2007). I-II grades tumours always tend to develop into relatively higher grades ones, and the infiltrative nature of GBM cells makes surgical resection incomplete and patients relapse soon. The key biological features of its malignant phenotype are characterized by non-limited tumour cell growth, resistance to pro-apoptotic stimuli, neovascularisation and evasion of anti-tumour immune surveillance (Giese and Westphal, 1996; Kleihues and Cavenee, 2000). Glioma cells acquire these malignant properties basically due to their secretion and activation of proteases, for example, serine proteases and metalloproteases (MPs), which results in degrading extracellular matrix and breaching natural barrier to invasion and metastasis to distant sites (Coussens and Werb, 1996; Rao, 2003).

1.2 Chemotherapy of glioma using temozolomide (TMZ)

Temozolomide (TMZ) combined with surgical resection and radiotherapy is the standard therapy for malignant glioma, however, the acquired chemoresistance of glioma cells limits its effect. TMZ (8-Carbamoyl-3-methylimidazo(5,1-d) -1,2,3,5-tetrazin-4(3H)-one) undergoes a non-enzymatic conversion at physiologic pH to its active complex 5-3-(methyl)-1-(triazen-1-yl)imidazole-4-carboxamide (MTIC),

which is rapidly converted to the inactive 5-aminoimidazole-4-carboxamide (AIC) and the electrophilic alkylating methyldiazonium cation that transfers a methyl group to DNA (Baker *et al.*, 1999; Villano *et al.*, 2009). A negligible percentage (1%-2%) of TMZ is metabolised to TMA, which is the carboxylic acid analogue of TMZ (Baker *et al.*, 1999; Tsang *et al.*, 1990). Alkylation of the O^6 position of guanine is the main contribution to the cytotoxic effect of TMZ. The methylated guanine mismatches with thymine in double-stranded DNA (O^6G-T) in the first cell-cycle after the treatment, which induces futile cycles of mismatch repair resulting in either double strand breaks or a critical recombinogenic secondary lesion. The secondary lesion is considered as an apurinic/athymidinic site formed in faulty mismatch repair that blocks replication causing either DNA double strand breaks, sister chromatid exchange or other abnormalities (Beier *et al.*, 2011; Kaina *et al.*, 1997; Karran and Bignami, 1994; Ochs and Kaina, 2000; Roos and Kaina, 2006). Therefore, a sufficient DNA repair system is necessary for TMZ to exert its cytotoxic effect on tumour cells (Beier *et al.*, 2011).

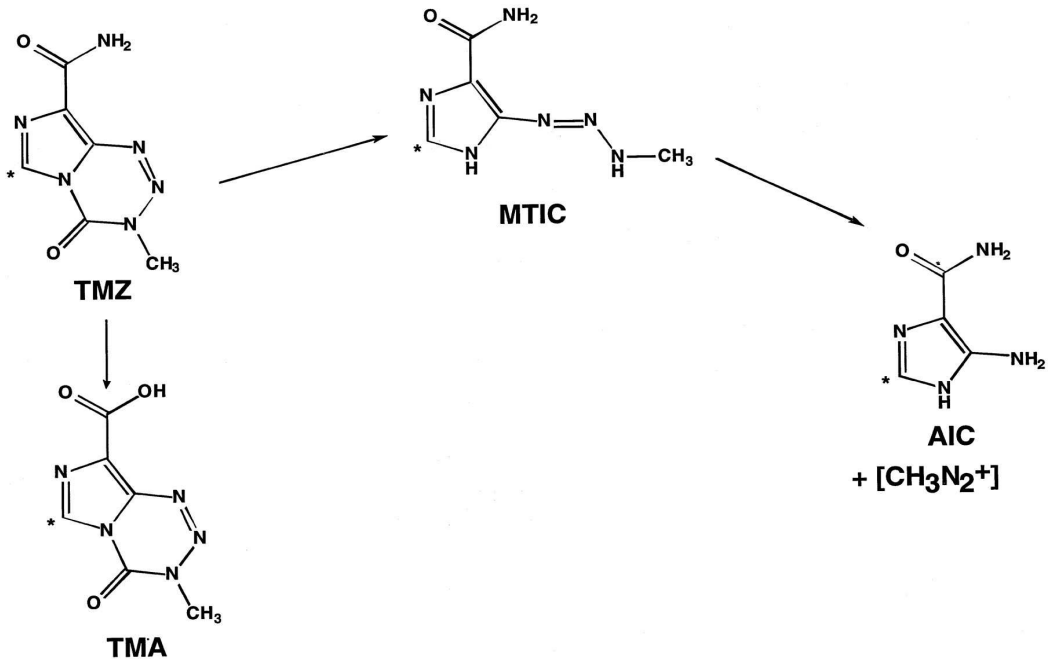

Figure 1. Chemical structure of TMZ and proposed metabolism and degradation pathways
*From: Baker SD, et al.(Baker *et al.*, 1999)

As a recent, oral, second generation alkylating agent, TMZ shows its efficacy for the

treatment of high-grade gliomas, which has been demonstrated in both pre-clinical and phase I- and II-studies (Dhodapkar *et al.*, 1997; Newlands *et al.*, 1992; O'Reilly *et al.*, 1993; Stupp *et al.*, 2002; Stupp *et al.*, 2005; Villano *et al.*, 2009). TMZ has favourable pharmacokinetic and pharmacodynamic properties. Moreover, because of its improved tolerability, overall survival and health-related quality of life in patients with malignant gliomas, TMZ appears to be an ideal, first-line, single agent for patients with high-grade gliomas (Villano *et al.*, 2009).

Even though TMZ combined with surgery and radiotherapy has been verified to prolong overall survival and improve the quality of life in patients with high-grade gliomas, a high prevalence of acquired resistance of glioma cells to TMZ becomes the major limitation in treatment of gliomas (Strik *et al.*, 2012), which is probably due to intrinsic cellular chemoresistance, excessive proliferation and enhanced invasive ability. Understanding the underlying mechanism of the resistance is very important. Until now, several mechanisms of TMZ resistance were unravelled: Oliva CR. and colleagues (Oliva *et al.*, 2011) reported that chemoresistance of gliomas to TMZ is related to a remodelling of the entire electron transport chain, with significant increases in the activity of complexes II/III and cytochrome C oxidase；Kitange GJ, et al (Kitange *et al.*, 2009) found that resistance to TMZ in GBM is modulated by the status of DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT), and elevated MGMT protein levels or lack of MGMT promoter methylation is associated with TMZ resistance in glioblastoma (Kitange *et al.*, 2009); Ulasov and co-workers showed that Sonic Hedgehog (SHH) and Notch pathway contribute to the chemoresistance of $CD133^+$ glioma stem cells to TMZ, and their antagonism results in an enhanced sensitivity when used in combination with TMZ (Ulasov *et al.*, 2011).

Expression of metalloproteases has been shown to be increased in gliomas and other cancer types and their expression levels relate to tumour severity and malignant biological behaviour. We therefore presume that up-regulated metalloproteases could contribute to the resistance of glioma cells to temozolomide therapy.

1.3 Matrix Metalloproteases (MMPs)

MMPs (Matrix Metalloproteases) constitute a family of zinc-dependent endopeptidases. About 23 MMPs are expressed in humans. The MMPs have been divided into collagenases, gelatinase, stromelysins and matrilysins according to their specificity for ECM (Extracellular Matrix) components. At present, they are categorised on the basis of their structure (Egeblad and Werb, 2002). Almost all MMPs share three common domains: the pre-domain, the prodomain, the catalytic peptide and the hemopexin-like C-terminal domain. MMPs are initially produced as an enzymatically inactive form, which is as a result of the interplay between a cysteine residue of the pro-peptide and the zinc-ion bound to the catalytic domain ("cysteine-switch"). Upon proteolytic removal of the prodomain or modification of the cysteine residue, the pro-MMPs are converted into active ones. Intracellular furin-like serine protease or some other extracellular activated MMPs as well as serine protease are involved in the activating process (Kessenbrock *et al.*, 2010; Sternlicht *et al.*, 1999).

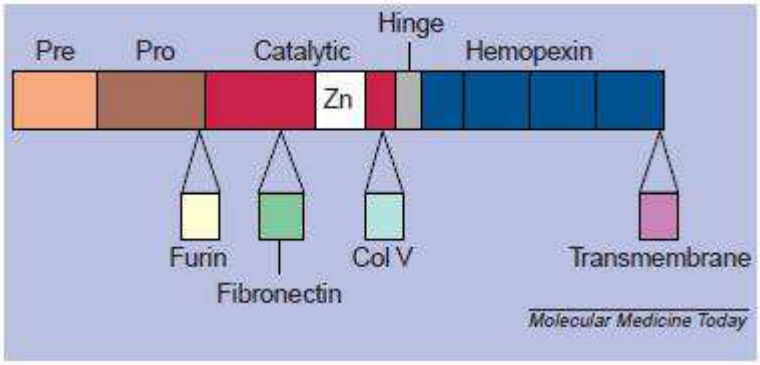

Figure 2. MMP domain structures
Most MMPs share four common domains: pre-domain, prodomain, catalytic domain and Hemopexin domain. Furin domain for activation of MMPs. Fibronectin and Col V domains for substrate recognition.
*From: McCawley LJ, et al.(McCawley and Matrisian, 2000)

The activities of MMPs *in vivo* are controlled by the local balance between themselves and their physiological inhibitors. α2-Macroglobulin (α2-MG), the main MMPs inhibitor in tissue fluid, is produced in liver and released into plasma and then

binds to MMPs (Sareddy *et al.*, 2009). The α2-MG-MMP complex is recognised by a scavenger receptor and irreversibly eliminated by endocytosis (Sottrup-Jensen and Birkedal-Hansen, 1989). The most important and best-studied endogenous inhibitors of MMPs are the tissue inhibitors of metalloprotease (TIMPs), which are also expressed in tumour lesions (Deryugina and Quigley, 2006). TIMP-1,-2,-3,-4 reversibly inhibits MMPs in a 1:1 stoichiometric form. The expression of TIMPs is tissue-specific and differs in their abilities to inhibit various MMPs. Some other factors, like local reactive oxygen species (ROS) (Weiss *et al.*, 1985), localization of MMPs (Nagase *et al.*, 2006), also mediate MMP activation and influence their biological functions.

MMPs play an important role in various physiological and pathological processes including tissue remodeling, organ development (Page-McCaw *et al.*, 2007), inflammatory courses (Parks *et al.*, 2004) and malignant diseases (Egeblad and Werb, 2002). Historically, MMPs are mainly considered to contribute to tumour cell invasion and metastasis by degrading almost all components of ECM (Liotta *et al.*, 1980). Nowadays, mounting evidence shows that their functions are more complicated than initially thought (Kessenbrock *et al.*, 2010). MMPs, as well as ADAMs, participate in the release of membrane-anchored latent forms of many growth factors (Peschon *et al.*, 1998). MMP-2 and MMP-9 are detected to induce TGF-ß (transforming growth factor-ß) activation and active TGF-ß could promote tumour growth by stimulating angiogenesis and suppressing immune surveillance (Yu and Stamenkovic, 2000). MMP function may disrupt the induction of apoptosis in malignant cells. Liu, et al showed that MMP-7 expression could help predict the chemoresistance in patients with non-small cell lung cancer (Liu *et al.*, 2008), which could be due to MMP-7 cleaving Fas ligand from the surface of tumour cells resulting in abrogating apoptosis (Mitsiades *et al.*, 2001). MMP-1 was observed to proteolytically cleave PARs (protease-activated receptors), which could affect tumour invasion by inducing cancer cell migration (Boire *et al.*, 2005). MMP-2, MMP-9 and MMP-14 are involved in tumour angiogenesis, and in fact, each MMP can contribute

to diverse vascular events in the tumour (Littlepage *et al.*, 2010). Moreover, MMPs and ADAMs also participate in modulating the function of cytokines produced by immune cells and inflammation processes, which are in many aspects linked to cancer progression (Lin and Karin, 2007).

In glioma samples, elevated expression of several MMPs, like for example MMP-1, MMP-2, MMP-7, MMP-9, MMP-11, MMP-12, MMP-14 and MMP-19, has been reported, which shows close correlation between their expression and tumour malignant progression *in vivo* (Forsyth *et al.*, 1998; Hur *et al.*, 2000; Stojic *et al.*, 2008; Tonn *et al.*, 1999). MMP-9, MMP-2 and its activator MMP-14 have been demonstrated to contribute to the active migration and invasion of tumour into normal brain tissue (Kargiotis *et al.*, 2008; Nakada *et al.*, 2003). MMP-2 inhibition in glioma cell lines, mediated by a plasmid vector carrying siRNA against MMP-2, decreased radiation-induced MMP-2 expression, cell viability and radiation-enhanced cell migration and invasion (Badiga *et al.*, 2011).

1.4 A Disintegrin and Metalloprotease (ADAM) family

Closely related to MMPs is the so-called ADAM family whose members belong to type I trans-membrane proteins. Until now, about forty gene members of the ADAMs are defined, of which only 21 are found functional in humans (Duffy *et al.*, 2011; Edwards *et al.*, 2008). The proteins share some similar domains composed of a prodomain, a metalloprotease domain, a disintegrin and integrin-binding domain, a cysteine-rich region, an EGF (epidermal growth factor)-like domain, a trans-membrane domain and an intracellular C-terminal domain. Similar to MMPs, ADAMs are also primarily produced as inactive precursor forms due to the interaction of a cysteine residue in the prodomain with the zinc ion at the catalytic site, and removal of the prodomain is prerequisite for activating the latent forms (Edwards *et al.*, 2008; Murphy, 2008). ADAMs play very important roles in cell adhesion, cell fusion, proteolytic processing of cell trans-membrane proteins and cell signalling (Blobel, 2005; Duffy *et al.*, 2009; Edwards *et al.*, 2008; Moss and Bartsch, 2004;

Murphy, 2008; Stupack, 2007). They contribute to extensive physiological and pathological processes in accordance with the changes of their expression. Especially in tumour development and progression, mounting evidence indicates that ADAMs proteins are involved in tumour occurrence, tumour cells invasiveness, cells migration and proliferation (Arribas *et al.*, 2006; Duffy *et al.*, 2009; McGowan *et al.*, 2008; McGowan *et al.*, 2007; Peduto *et al.*, 2006; Roemer *et al.*, 2004). For instance, ADAM10 is demonstrated to promote the tumour progression by cleaving membrane-bound proteins including Notch molecule (Xu *et al.*, 2010). The expression of ADAM9 is very low in normal epithelium of uterine cervix, but significantly increased in squamous cell carcinomas (SCC) of the uterine cervix (Zubel *et al.*, 2009). L1CAM (CD171, a trans-membrane protein involved in cell adhesion, survival, growth, migration and invasion) is specifically cleaved by ADAM10 and Presenilin resulting in its translocation to nuclei to regulate gene expression, DNA damage response and radiosensitivity of glioblastoma stem cells (Cheng *et al.*, 2011; Riedle *et al.*, 2009). Compared to non-neoplastic brain tissue, the expression of ADAM17 is four times more increased and correlates closely to the malignant phenotypes of glioma cells (Zheng *et al.*, 2012).

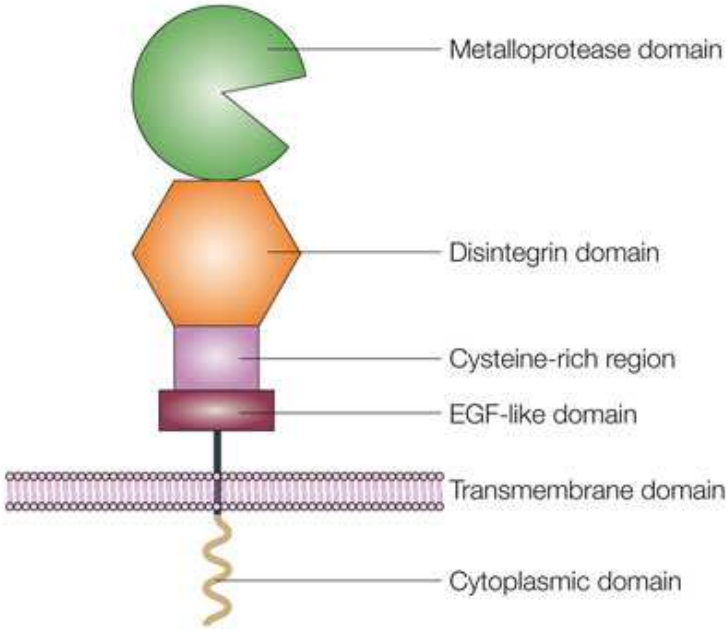

Figure 3. Structure of mature form of ADAM.
A typical ADAM protein contains the following domains: a metalloprotease domain, a disintegrin domain, a cysteine-rich region, an epidermal growth factor (EGF)-like domain, a trans-membrane domain and a cytoplasmic domain. Additionally, latent ADAMs contain an N-terminal signal sequence and a prodomain, which are not shown.
*From: Blobel CP (Blobel, 2005)

ADAM8, a member of human ADAM family, is a membrane-bound protein composed of 824 amino acids that is coded by a gene positioned on chromosome 10q26.3 (Hall *et al.*, 2009). Different from typical furin-mediated activation of ADAMs, ADAM8 is autocatalytically activated. Similar to major amount of trans-membrane proteins, ADAM8 is produced as a potential form. Propeptide removal is necessary for the activation, and the disintegrin/cysteine-rich domain and metalloprotease peptide play crucial roles in the removal of prodomain (Schlomann *et al.*, 2002). After removal of the prodomain, ADAM8 molecule is localized in the cell membrane as an active sheddase (mature form), which is further processed into a soluble metalloprotease and a remnant protein consisting of the DIS, Cys, EGF-like, TM and CD domain. The remnant form is found more abundant than the precursor

and mature forms in human ADAM8 (Schlomann *et al.*, 2002). The soluble MP which is not inhibited by any of the TIMP proteins cleaves ECM protein consistent with the invasive function of ADAM8 (Ishikawa *et al.*, 2004; Valkovskaya *et al.*, 2007; Wildeboer *et al.*, 2006).

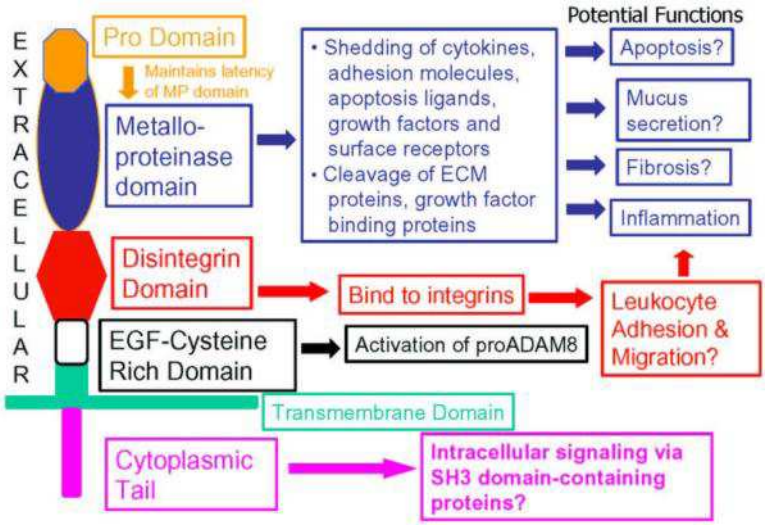

Figure 4. Structure ＆ potential functions of ADAM8 in the organism
*From: Knolle MD, et al. (Knolle and Owen, 2009)

ADAM8 is widely expressed in the immune system (Kataoka *et al.*, 1997; Richens *et al.*, 2007), bone cells (Ainola *et al.*, 2009; Choi *et al.*, 2001), lung (King *et al.*, 2004), ovary (Sriraman *et al.*, 2008), salivary glands, kidney (Kelly *et al.*, 2005) and the central nervous system (Schlomann *et al.*, 2000). In the central nervous system (CNS), ADAM8 expression is generally low, however, it is significantly increased in the inflamed CNS, in reactive astrocytes, oligodendrocytes, activated microglia, and in degenerating neurons, and exerts a neuro-protective effect (Bartsch *et al.*, 2010; Mahoney *et al.*, 2009; Schlomann *et al.*, 2000). In several cancer types, ADAM8 has been shown to correlate with tumour severity and invasiveness of tumour cells. For instance, the expression level of ADAM8 is extensively up-regulated in glioblastoma tissues and consistent with the tumour progression and bleak prognosis of patients (He *et al.*, 2011). Fritzsche, et al found the over-expression of ADAM8 in prostate cancer tissues and its highly increased expression is compatible with the dismal prognosis of

patients (Fritzsche *et al.*, 2006). Wilderboer, et al observed maximal ADAM8 mRNA expression in glioblastoma, low-grade oligoastrocytoma and anaplastic ependymoma. The abundant remnant form of ADAM8 was detected in all astrocytic tumours, which implied that most ADAM8 molecules existed as an activated form in the tumours. Furthermore, invasiveness assays revealed that up-regulation of ADAM8 resulted in promoted migration or invasive activity of glioma cells (Wildeboer *et al.*, 2006).

1.5 Effect of presenilin (PS)/γ-secretase

Since presenilin (PS)-dependent γ-secretase (PS/γ-secretase) was primarily discovered as an enzyme complex involved in generation of amyloid-ß (Aß), which is the main contributor to Alzheimer's disease (AD), more than 90 additional proteins have been detected to undergo similar proteolytical processing by this enzyme (Haapasalo and Kovacs, 2011). PS/γ-secretase and site-2 protease (S2P), along with signal peptide peptidases (SPPs) and rhomboids consist of a family of intramembrane cleaving proteases (Weihofen and Martoglio, 2003), and they are separately responsible of cleaving diverse membrane-anchored proteins and involved in regulation of different cellular events.

PS/γ-secretase is an enzyme complex with its molecular weight varying between 250 and 2000 kDa (Haapasalo and Kovacs, 2011). In addition to presenilin, three other proteins have been identified as the components of active γ-secretase complex: Aph-1, a multipass trans-membrane protein, a type-I trans-membrane protein nicastrin and Pen-2, a membrane protein enhancing PS function. The four core components form the active PS/γ-secretase in 1:1:1:1 stoichiometry. Moreover, they are interdependent and interrelate with each other even though each of them has their special tasks (Sato *et al.*, 2007; Spasic and Annaert, 2008; Vetrivel *et al.*, 2006). Presenilins (presenilin-1 and presenilin-2 encoded by PSEN1 and PSEN2, respectively) are key components of γ-secretase protease containing the catalytic site of the enzyme complex.

Most γ-secretase substrates are type-I trans-membrane proteins and its cleaving

membrane-anchored proteins stubs occurs generally after the ectodomain shedding of full-length substrates (Struhl and Adachi, 2000). Furthermore, these substrates are involved in various cellular events, for example cells proliferation and apoptosis, adhesion, cells migration, neurite outgrowth. The intracellular domains (ICDs) of several substrates trans-locate into nucleus and play a role in transcriptional regulation of their downstream target genes after released by PS/γ-secretase. And the PS/γ-secretase mediated cleavage of ICDs functions as a regulatory switch in signalling of these special substrates. For instance, Notch family proteins regulate gene transcription via Notch activation and subsequent cleavage of Notch-ICD by γ-secretase, by which Notch proteins control cell-fate decisions and maintenance of stem cell populations during development (Haapasalo and Kovacs, 2011). PS/γ-secretase activity has also been implicated in the regulation of angiogenesis and tumourigenesis. Cai and colleagues found that vascular endothelial growth factor receptor-1 (VEGFR-1) underwent similar regulated intramembrane proteolysis (RIP) by γ-secretase resulting in the inhibited angiogenesis (Cai *et al.*, 2006). Pelletier L, et al demonstrated that γ-secretase participate in the release of CD144-ICD leading to promoted cell transformation (Pelletier *et al.*, 2006). As an important endogenous protease, PS/γ-secretase combined with metalloproteases (MPs) is suggested to be involved in DNA damage response (DDR) and an important underlying mechanism of the resistance of glioma cells to TMZ.

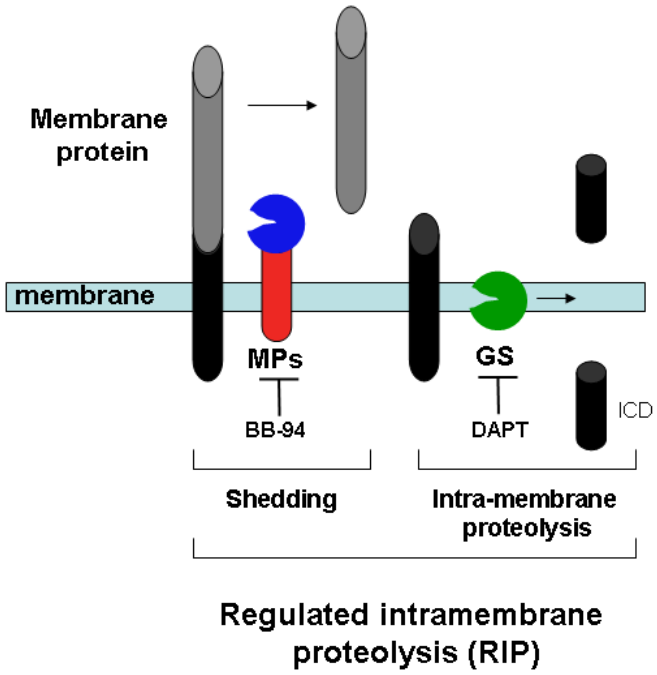

Figure 5. Metalloproteases (MPs) and gamma-secretase (GS) involved in processing of trans-membrane proteins
MPs (ADAMs and some MMPs) and GS (γ-secretase/PS, presenilins) involved in the processing of trans-membrane proteins. MPs cleave the ectodomains, and afterwards, γ-secretase participates in the releasing of ICDs. BB-94 and DAPT function as an inhibitor of MPs and γ-secretase, respectively.

1.6 BB-94 (Batimastat) inhibitor and DAPT inhibitor

BB-94 (molecular formula: $C_{23}H_{31}N_3O_4S_2$) is a well-known synthetic inhibitor of metalloproteases containing a peptide domain mimicking the cleavage site of collagen and a hydroxamate group, which is bound by metalloproteases and the zinc ion in the catalytic site of the metalloprotease, respectively. The metalloprotease is thereby inactivated. Since the majority of MPs share a homologous catalytic domain, BB-94 exhibits a broad-spectrum inhibition property (Hoekstra *et al.*, 2001)

DAPT (molecular formula: $C_{23}H_{26}F_2N_2O_4$) is widely used as a novel γ-secretase inhibitor, which is usually applied in research to inhibit generation of amyloid-β and in clinical trials to treat Alzheimer's disease (AD). DAPT inhibits proteolytic activity

of γ-secretase in a non-competitive manner by an unknown mechanism. Barthet et al showed that DAPT stabilizes interplay between presenilin-CTF, the key component of γ-secretase complex, and other partner components, the APH-1/nicastrin and PS1-NTF/PEN-2 subcomplexes, resulting in a tensed conformation of γ-secretase enzyme complex. The stabilization effect of DAPT is a potential mechanism by which the drug regulates γ-secretase activity (Barthet *et al.*, 2011).

2 Aims of the study

Understanding the potential molecular mechanism contributing to the resistance of glioma cells to the standard drug TMZ is important for optimizing existing or developing novel therapeutic strategies. It is reported that ADAM10 is involved in resistance of glioma cells by regulating L1CAM shedding that decreases radiosensitivity of glioblastoma stem cells (Cheng *et al.*, 2011; Riedle *et al.*, 2009), and MMP-2 inhibition sensitizes glioma cells to radiotherapy (Badiga *et al.*, 2011). Furthermore, expression of metalloproteases (MPs) in glioma is remarkably up-regulated and associated with the malignant phenotype of glioma cells. We hypothesized that MPs (ADAMs and/or MMPs) contribute to resistance of glioma cells against TMZ by cleavage of membrane-bound proteins or increasing tumour cells proliferation and/or invasiveness.

3 Materials

3.1 Cell lines

U87-MG glioblastoma cell line was bought from American Type Culture Collection (ATCC).

3.2 Media and solutions for cell culture

U87-MG cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) High Glucose (4.5g/l) supplemented with 1% L-Glutamine (200mM), 1% Penicillin/ Streptomycin 100×, 1mM sodium pyruvate solution, 1% MEM Non Essential Amino Acids 100× and 10% Fetal Bovine Serum (heat inactivated).

For activity assays, complete growth medium was exchanged into DMEM High Glucose (4.5g/l) without serum and phenol red on the day before collecting the supernatant and harvesting the cells.

All materials above were bought from PAA Laboratories GmbH.

3.3 Kits

Description	Manufacturer
PeqGOLD Total RNA Kit	PEQLAB Biotechnologie GmbH, Erlangen
RNA to cDNA EcoDry™ Premix (Double Primed)	Clontech Laboratories, Inc. A Takara Company
Pierce® BCA Protein Assay Kit	Thermo scientific, USA
SuperSignal® West Femto Maximum Sensitivity Substrate	Thermo scientific, USA

3.4 Antibodies

Antibody	Dilution	Manufacturer
First antibodies		
Anti-ADAM8 cytoplasmic domain (rabbit polyclonal)	1:2000	Millipore, USA
Anti-hMMP-2 affinity purified goat IgG	1:1000	R&D systems
Anti-hMMP-9 affinity purified goat IgG	1:1000	R&D systems
Anti-MMP-14 rabbit monoclonal antibody	1:1000	Epitomics-Abcam Company, USA
Anti-p-p44/42 MAPK (T202/Y204) rabbit Ab	1:2000	Cell Signaling Technology
Anti- ß-Tubulin (H-235) rabbit polyclonal	1:500	Santa Cruz Biotechnology
Secondary antibodies		
Anti-goat IgG, HRP-conjugated	1:2000	R&D systems
Anti-rabbit IgG, HRP-linked antibody	1:2000	Cell Signaling Technology

3.5 Protein ladder

PageRuler™ Plus Prestained Protein ladder is manufactured by Pierce Biotechnology, Thermo Scientific, USA.

3.6 Buffers and Solutions

Buffer and Solutions	Ingredients
Renaturing buffer	3% Triton X-100 in ddH_2O
Developing buffer	50mM Tris PH 7.5, 200mM NaCl, 5mM $CaCl_2$, 0.02% Brij-35 in ddH_2O
Coomassie staining buffer	50% Methanol p.A., 10% Acetic Acid, 0.5% Coomassie Brilliant Blue in ddH_2O
Coomassie destaining solution	50% Methanol p.A., 10% Acetic Acid in ddH_2O
2×Sample buffer (non reducing)	2.5ml 0.5M Tris-HCl PH 6.8, 2ml Glycerol, 4ml 10% SDS, 0.5ml 0.1% Bromphenol Blue to 10ml with ddH_2O
RIPA buffer	50mM HEPES PH 7.4, 150mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS+1×complete protease inhibitor, 1×Phosstop
5×loading buffer	3.125ml 1M Tris PH 6.8, 1g SDS, 4.5ml Glycerol, 125µl 1% BPB, 1.25ml ß-Mercaptoethanol
10×Running buffer	144g Glycine, 30g Tris Base, 10g SDS to 1L with ddH_2O
10×Transfer buffer	144g Glycine, 30g Tris Base to1L with ddH_2O
1×Transfer buffer (Western Blot)	100ml 10×Transfer buffer, 200ml Methanol to 1L with ddH_2O
5% Milk blocking solution	5g no-fat dry milk powder, 100µl Tween 20, 100ml PBS (1×)
Substrate assay buffer	1ml 1M Tris PH 8.0, 500µl 1M $CaCl_2$, 50µl 1mM $ZnCl_2$, 1µl Brij® 35 solution to 50ml with ddH_2O
MTT solvent	5ml 1M HCl, 5ml Triton X-100, 40ml Isopropanol

3.7 Drugs and inhibitors

Drugs and inhibitors	Description	Stock concentration	Manufacturer
Temozolomide (TMZ)	alkylating agent for high-grade glioma	100mM	Sigma Life Science
Batimastat	broad-range metalloprotease inhibitor	500µM	a kind gift from Dr. M. Zack (Pfizer Global R&D, Chesterfield, MO, USA
MTT	dimethylthiazol-diphenyl tetrazolium bromide	0.5mg/ml	Sigma Life Science
DAPT	N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester γ-secretase inhibitor	10mM	Sigma Life Science
UO126	A dual pERK1/ERK2 inhibitor	20mM	Source Bioscience

3.8 Oligonucleotides used for qRT-PCR

All primers were synthesized by APARA-Bioscience (Freiburg, Germany) and were dissolved in nuclease-free water in a stock concentration of 100µM.

hMMP-1 fw 5’-CGG TTT TTC AAA GGG AAT AAG TAC-3’

hMMP-1 rev 5’-TCA GAA AGA GCA GCA TCG ATA TG-3’

hMMP-2 fw 5’-GCG GCG GTC ACA GCT ACT T-3’

hMMP-2 rev 5’-CAC GCT CTT CAG ACT TTG GTT CT-3’

hMMP-7 fw 5’-CTG GAC GGA TGG TAG CAG TC-3’

hMMP-7 rev 5’-CAT ACC CAA AGA ATG GCC AAG-3’

hMMP-9 fw 5’-ACC TCG AAC TTT GAC AGC GAC-3’

hMMP-9 rev 5’-GAG GAA TGA TCT AAG CCC AGC-3’

hMMP-14 fw 5’-GGC TAC AGC AAT ATG GCT ACC-3’

hMMP-14 rev 5’-GAT GGC CGC TGA GAG TGA C-3’

hMMP-16 fw 5’-CTG CGG AAC GGA GCA GTA TTT-3’

hMMP-16 rev 5’-GGT AGC CGT AGT TTT GTA ACC A -3

hMMP-17 fw 5’-CAC TCA TGT ACT ACG CCC TCA-3’

hMMP-17 rev 5’-TGG AGA AGT CGA TCT GGA TGT C-3’

XS 13 fw 5’-TGG GCA AGA ACA CCA TGA TG-3’

XS 13 rev 5’-AGT TTC TCC AGA GCT GGG TTG T-3’

hADAM8 fw 5’-ACA ATG CAG AGT TCC AGA TGC-3’

hADAM8 rev 5’-GGA CCA CAC GGA AGT TGA GTT-3’

hADAM9 fw 5’-TCC ATT GCT CTT AGC GAC TGT-3’

hADAM9 rev 5’-GGT TCA ATC CCA TAA CTC GCA T-3’

hADAM10 fw 5'-ATG GGA GGT CAG TAT GGG AAT-3'

hADAM10 rev 5'-ACT GCT CTT TTG GCA CGC T-3'

hADAM12 fw 5'-CGA GGG GTG AGC TTA TGG AAC-3'

hADAM12 rev 5'-GTC CCC ACT CCC AAC AGA G-3'

hADAM17 fw 5'-GTG GAT GGT AAA AAC GAA AGC G-3'

hADAM17 rev 5'-GGC TAG AAC CCT AGA GTC AGG-3'

hADAM19 fw 5'-GGG AGC CTG GAT GGA CAA G-3'

hADAM19 rev 5'-AGC TTT GAG TGG ATG CTT TTC TC-3'

3.9 qRT-PCR reagents

For each well: 10µl SensiFast SYBR Hi-ROX mix (2×), 0.4µl forward primer, 0.4µl reverse primer, 7.2µl nuclease-free water and 2µl cDNA sample

SYBR master mix was bought from Bioline Ltd, UK.

4 Methods

4.1 MTT assay

With cellular NAD-dependent dehydrogenase in living cells cleaving the tetrazolium ring, MTT (dimethylthiazol-diphenyl tetrazolium bromide) is converted into insoluble purple formazan crystals, which is solubilised by isopropanol and the dehydrogenase activity is determined spectrophotometrically by measuring absorbance at 570nm. The amount of the purple formazan crystal relates to the number of viable cells. The assay is therefore convenient and the common method for measuring viability of cells.

After cells have been cultured under different conditions in 96-well plates for several days, medium was aspirated and 100µl MTT solution was added per well followed by incubation at 37^{o}C, 5%CO_2 for 4 hours. Afterwards, 100µl MTT solvent was added in each well to end the reaction. Pipetting up and down completely dissolved, the crystals and absorbance values (λ=570nm) were determined in a 96-well-plate reader (BMG Labtech, Offenburg, Germany).

4.2 Real-time quantitative polymerase chain reaction (qPCR)

The transcriptional regulation of selected genes was analysed using quantitative real-time PCR (Applied biosystem StepOne™ and StepOnePlus™ real-time PCR system). Real-time PCR (qPCR) is a method based on the detection and quantification of a fluorescent reporter signal that increases in direct correlation to the amount of the PCR product in the reaction (Lee *et al.*, 1993; Livak *et al.*, 1995). The cDNA was detected and quantified with the fluorescent dye SYBR Green, which offers a linear dose response over a wide range of target concentrations. As cDNA accumulates, the dye produces a signal that is proportional to the cDNA concentration. ROX reference dye was used to normalize the fluorescent signal between reactions. qPCR reactions were performed in 20 µl volume by using the qPCR mix.

The program was run as follows:

2-step cycling:

Cycles	Temperature	Time	Notes
1	95°C	2 min	Polymerase activation
40	95°C 60°C	5 s 15 s	Denaturation Annealing/extension

Formation of a single specific PCR product was confirmed by melting curve analysis. Acidic ribosomal protein XS13 whose expression is at the same level in cells served as an internal concentration reference gene for all real-time PCR reactions (personal communication, Dr. M. Buchholz, Marburg University). Relative changes in gene expression were determined with the ΔCt method using the following formula: ΔCt = (Ct reference – Ct target). Differential gene expression between conditions corresponds to the log2 fold-difference in mRNA levels between the conditions compared (Livak and Schmittgen, 2001).

4.3 Protein analysis

4.3.1 Determination of cells number

To determine the cell number of the cell suspension after trypsination, 10µl single-cell suspension was transferred into a Neubauer hemocytometer. The cell numbers in each of the outer squares were counted and averaged to obtain a mean value. The mean cell number was multiplied by the factor 10^4 to obtain the number of cells per milliliter.

4.3.2 Sample preparation

U87-MG cells were plated in 10cm dishes and treated with chemotherapy drugs and/or inhibitors for 3days, 5days and 7days, respectively. For collecting the

supernatants, complete culture medium was exchanged by DMEM High Glucose without serum and phenol red 24 hours before analysis. The conditioned DMEM was collected and centrifuged at 4,000 rpm for 10minutes to eliminate cell debris. Fresh supernatants were required for protein activity assays, while for Western Blot the samples were stored at -80^{o}C before use.

For preparing a protein lysate from cells, the culture medium was aspirated and RIPA buffer 600µl/dish was added followed by incubation at 4^{o}C 15 minutes. Cell scrapers were used to collect the lysates. The samples were sonicated and centrifuged at 13,000 rpm 5 minutes to remove the cell debris.

4.3.3 BCA protein assay

BCA protein assay was applied to measure the protein concentration of samples. Firstly, samples were diluted 1:5 and 1:10 with ddH_2O and standard Bovine Serum Albumin (BSA) test tubes (the original Albumin solution is produced by Thermo Scientific with a stock concentration of 2000µg/ml) were prepared as follows:

vial	Volume of diluent	Volume and source of BSA	Final BSA concentration
A	0	300µl of stock BSA(2mg/ml)	2000µg/ml
B	125µl	375µl of stock BSA	1500µg/ml
C	325µl	325µl of stock BSA	1000µg/ml
D	175µl	175µl of vial B	750µg/ml
E	325µl	325µl of vial C	500µg/ml
F	325µl	325µl of vial E	250µg/ml
G	325µl	325µl of vial F	125µg/ml
H	400µl	100µl of vial G	25µg/ml

The standard and unknown samples, along with the blank sample were added into a

96-well-plate in duplicate. Pierce® BCA protein assay kit was used to detect proteins in the samples. After incubation at 37°C for 30 minutes, the plate was read in a plate reader and the values for protein concentrations were obtained.

4.3.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins with different sizes were separated using SDS-PAGE, which is divided into non-reducing and reducing SDS-PAGE. Migration velocity of proteins in an electric field of proteins is proportional with their molecular weights. With samples and gel containing detergent SDS (sodium dodecyl sulfate) or reducing agent ß-mercapto-ethanol, the hydrogen bonds and disulfide bonds are broken and the secondary and tertiary protein structures are thereby reduced. Separated polypeptide chains combine with SDS, which results in proteins loaded with more negative charges than its original form and making all proteins moving to the positive pole in the electric field independent of their original shape.

Gel	Ingredient
Stacking gel	30% Acrylamide 0.333ml 1M Tris PH 6.8 0.25ml ddH_2O 1.385ml 10% SDS 20µl 10% APS 20µl TEMED 2µl
Separating gel	30% Acylamide 1.667ml 1M Tris PH 8.8 1.875ml ddH_2O 1.364ml 10% SDS 50µl 10% APS 40µl TEMED 4µl For Zymography, the substrate, either 0.1% Gelatin or 1mg/ml Casein , was added to the gel

4.3.5 Zymography assays

Zymography on the basis of SDS-PAGE is used to detect the activity of native MMPs. Samples were prepared in non-reducing sample buffer without boiling before the experiment and the substrate (either Gelatin or Casein) was added in a separating gel to co-polymerize with polyacrylamide. During electrophoresis, proteins are separated in the polyacrylamide gel and meanwhile SDS in the gel keeps MMPs in an inactive state. After the run, gels were washed with renaturing buffer (2×30 minutes each) afterwards resulting in partially renatured MMPs and restored their activities. Subsequently, the gel was incubated in developing buffer (1×30 minutes at room temperature and then at 37^{o}C in new developing buffer for 24hours) and the renatured MMPs in the gel digested the substrate. The next day, the gel was dyed in Coomassie staining buffer for 1 hour followed by Coomassie destaining solution (see page 25.),

so that the bands of MMPs are visualised against a blue background of undigested substrate.

4.3.6 Western Blot

Western Blot is a routine method to determine the expression status of proteins. Distinct from zymography assays, samples are prepared in the 5×loading buffer which contains detergent SDS and a reducing agent. Samples were heated at 95^{o}C for 10 minutes before electrophoresis. The secondary and tertiary structure of proteins are thereby reduced with losing their activities. After electrophoresis, separated proteins are transferred from the gel to a nitrocellulose membrane. Detection of target proteins is performed using specific antibodies (listed in 3.4 antibodies). A secondary antibody linked HRP (Horseradish peroxidase) is then used to cleave a chemiluminescent substrate and produces luminescence proportional to the amount of protein. Exposing the membrane to a luminescence reader (Intas, Chemostar Imager) provides an image of the proteins bound to the blot.

4.3.7 Protease activity assays

Synthetic polypeptide protein substrates have been greatly developed to estimate the protein activity in a real-time manner. These substrates are typically composed of a fluorescence resonance energy transfer (FRET) donor and a quencher fluorophore, which are linked by a sequence of amino acid containing a protease cleavage motif. Once the polypeptide is cleaved by the protease, the fluorophore donor separates from the quencher resulting in an increase of fluorescence. Thus, protease activity dynamics can be followed by tracking the changes in fluorescence over time (Moss and Rasmussen, 2007).

Within the last 24 hours of culturing cells under different conditions, the cells were maintained in DMEM High Glucose without L-Glutamine and serum. Supernatants were freshly collected and concentrated 10:1 using VIVASPIN 4 (cutoff 10kDa,

Sartorius Stedim Biotech.). PEPDAB013 (Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln –Lys-Set-Lys(5-FAM)-NH_2) and PEPDAB008 (Dabcyl-Pro-Cha-Gly-Cys(Me)His -Ala-Lys(5-FAM)-NH_2) produced by BioZyme Inc. were used as the specific substrates for ADAM8 and MMP-1, respectively. The substrates were stored in DMSO at -20 ^{o}C in a stock concentration of 10mM. After diluting the substrates 1:500 with substrate assay buffer, we added 20µl concentrated supernatants and 30µl substrate assay buffer along with 50µl diluted substrates per well in triplicate in a 96-well-plate. For blank wells, 50µl substrate assay buffer and 50µl diluted substrate solution were added per well. Fluorescence was measured every 2 minutes with excitation and emission wavelengths of 485 and 530nm, respectively. All data were normalized to identical cell numbers.

4.4 Invasion assay

The invasion assay provides a system in vitro to assess the invasive ability of U87-MG cells and the changes in invasiveness could be a result of the increase of metalloproteases expression, which could be a potential mechanism of chemoresistance of glioma cells.

The required number of inserts (Cell Culture Inserts 24-well 8.0µM pore size, BD Falcon™) were transferred into the wells of a 24-well plate with a sterile forceps and 75µl Matrigel (MATRIGEL™ MATRIX Basement membrane Matrix, BD Bioscience) was carefully pipetted into each insert. The 24-well plate was then incubated at 37^{o}C incubator for gelling. Cell suspension was prepared in serum-free DMEM containing 5000 cells/300µl for each invasion chamber. 750µl complete DMEM was added into each lower chamber as a chemoattractant and the invasion chambers were incubated in a humidified tissue culture incubator at 37^{o}C 5%CO_2 atmosphere for 24 hours. A cotton swap was then used to remove non-invading cells. The invading-cells on the lower surface were fixed with 4% formaldehyde for 15 minutes and stained with Hematoxylin solution (Carl Roth GmbH+Co. KG Germany) at room temperature for

5 minutes, washed with ddH_2O (3×10 minutes each time) and then counted under a light microscope.

4.5 Statistical analysis

All the experimental data were analysed using one-way ANOVA: Values are expressed as means ± SEM of n independent observations unless indicated otherwise. Statistical significance was evaluated by using paired student's t-test. Only for evaluation of inhibitor data (UO126) a two-way ANOVA was performed.

5 Results

5.1 Dose- and time-dependent effect of TMZ on U87-MG cells

U87-MG cells were treated with different concentrations of TMZ for 3 days, 5 days and 7 days, respectively. After incubation with TMZ for 3 days, about 70% cells survived even with TMZ of high concentrations in comparison with control. However, after exposure to TMZ (500μM or 700μM) for 5 days, only 50% viable cells were detected. After long-term incubation (up to 7 days) with TMZ, there was no significant difference in cells survival between different concentrations of TMZ and when compared to control, about 30% viable cells still remained. Our results implied that TMZ exerts its inhibition effect on U87-MG cells in a dose- and time-dependent manner (Fig 6.).

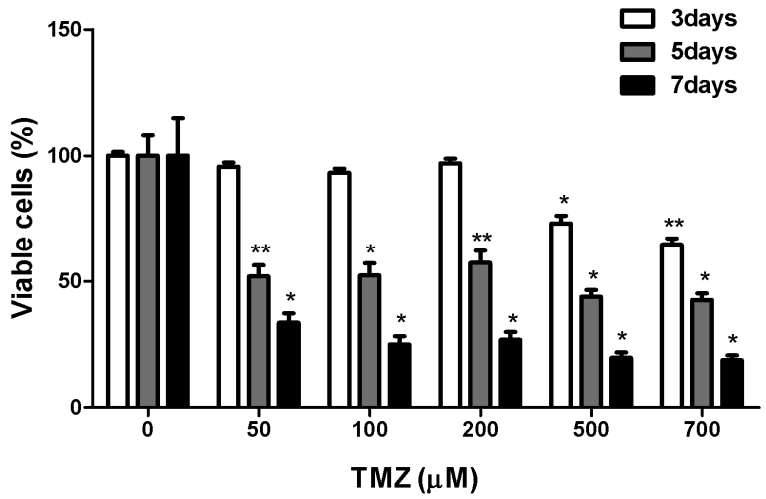

Figure 6. MTT assay to test effect of TMZ on the proliferation of U87-MG cells
TMZ exerted its inhibition effect on U87-MG cells proliferation in a dose–dependent manner. However, even after long-term incubation (up to 7 days) with high concentrations of TMZ, there were still about 30% viable cells left. Values are presented as mean, SEM, n=3. Significant differences between diverse concentrations of TMZ and control (0μM) at the same time point, paired t-test: *p<0.05, **p<0.01.

5.2 Induction of metalloproteases by TMZ in U87-MG cells

5.2.1 Induced expression of MPs by TMZ at mRNA level

Quantitative real-time PCR was used to demonstrate that TMZ induced the expression of metalloproteases (ADAMs or MMPs) at mRNA level. We found that incubation with TMZ for 3 days has not resulted in differences in expression levels of ADAMs and MMPs, whereas for 5 days, TMZ caused an increased expression of ADAM8, MMP-1, MMP-9 and MMP-14. Compared to control, the expression of ADAM8 was around 14-fold increased, and the difference was significant with $p < 0.01$. In addition, MMP-1, MMP-9 and MMP-14 were around 6-fold, 5-fold and 4-fold up-regulated, respectively. Incubation with TMZ has not led to significant increase of expression levels of other MPs analysed, even for 5 days (Fig 7.).

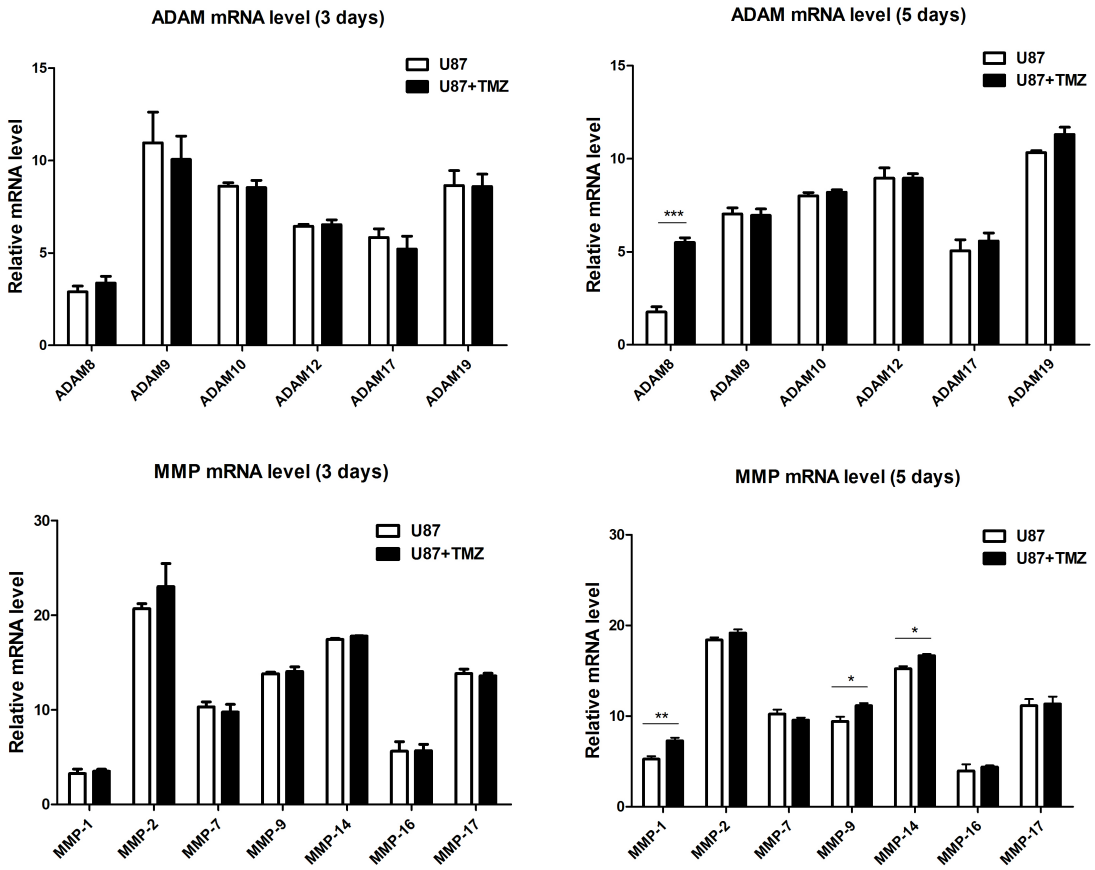

Figure 7. Differences in expression levels of MPs induced by TMZ determined by quantitative real-time PCR analysis

After incubation with TMZ for 3 days, no difference in expression of MPs between control and TMZ-treated cells was seen. Treatment of TMZ for 5 days resulted in significantly higher expression levels of ADAM8, MMP-1, MMP-9 and MMP-14 compared to control. Values are presented as mean, SEM, n=4. Significant differences, paired t-test: *p<0.05, **p<0.01, ***p<0.001.

5.2.2 Western blot of MPs induced by TMZ

Western blot was employed to detect protein concentrations and the processing status of ADAMs and MMPs under different culture conditions. The antibody against ADAM8 cytoplasmic domain revealed two bands of 120 and 90 kDa, representing the proform and mature form of ADAM8 which is the catalytically active form. This active form was more abundant compared to control after treatment of TMZ (500μM) for 5 days, and the degree of activation, determined by the relative proportion of the 90 kDa band to 120 kDa proform band, has been significantly enhanced after

treatment of TMZ, implicating that incubation with TMZ led to increased expression and activity of ADAM8 in U87-MG cells. MMP-14 western blot resulted in a 55kDa band (active form of MMP-14). Similar to the active ADAM8 band, U87-MG cells treated with TMZ for 5 days revealed an more intense band of MMP-14 compared to control. The antibody against ß-tubulin was used to normalize for protein loading. Concentrated supernatant of U87-MG cells under different culture conditions was applied to detect secreted MMP-9. The latent form of MMP-9 was found as a 92 kDa band. Consistent with the qPCR data, the expression level of MMP-9 in U87-MG cells was significantly induced after exposure to TMZ for 5 days. However, the expression of MMP-2, which is expressed highest in U87-MG cells among the surveyed MMPs in our study, was not detected to be induced by TMZ.

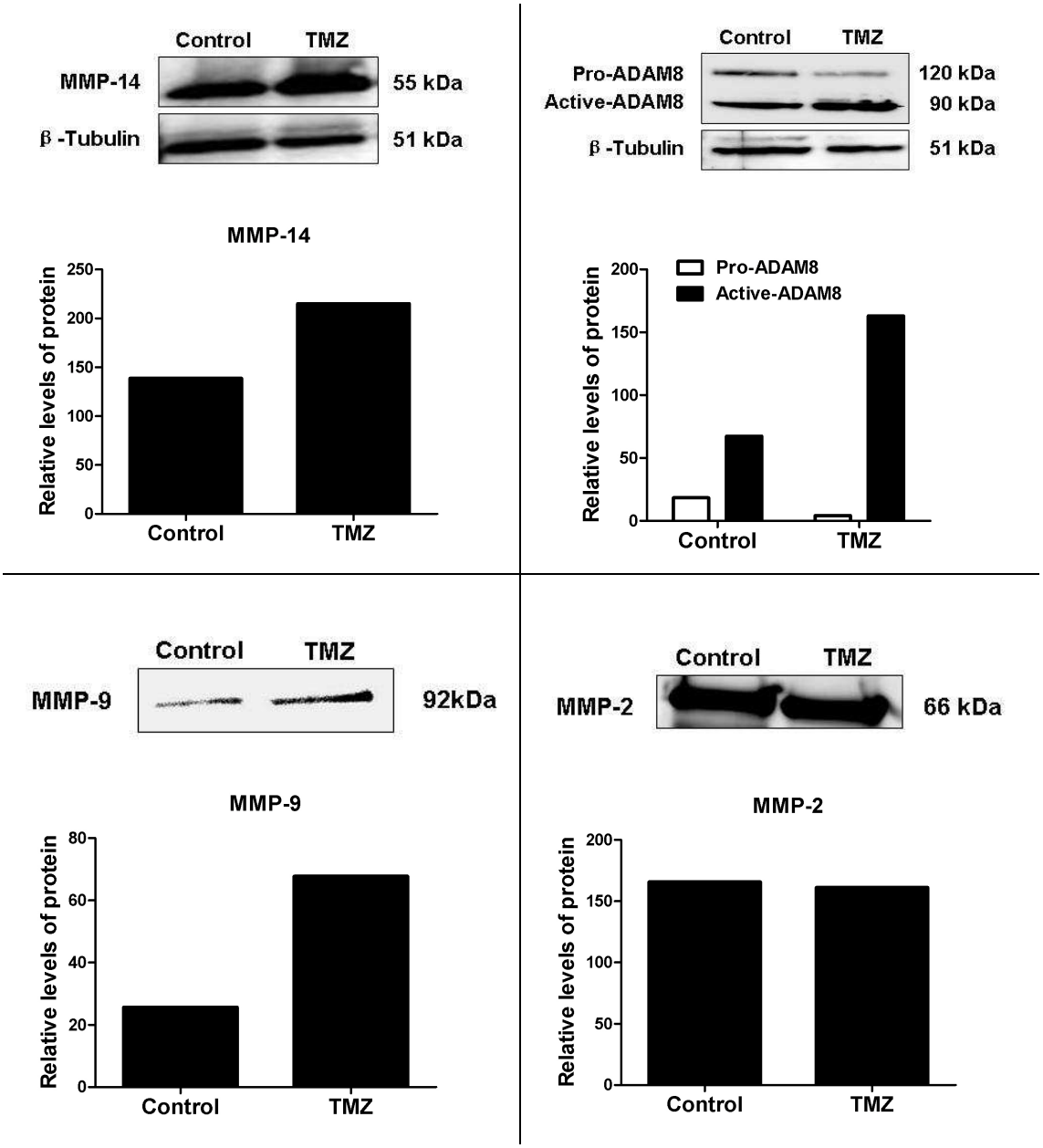

Figure 8. Western blot analysis of ADAM8, MMP-14 and MMP-9 in U87-MG cell protein lysate and supernatant samples, respectively

Protein concentrations were determined using BCA protein assay and equal amount of protein was added in each lane. Incubation with the antibody against ß-tubulin was performed to determine equal protein loading for cell lysate samples. After treatment of TMZ (500µM) for 5 days, more abundant active MMP-14 and ADAM8 and latent MMP-9 were detected in U87-MG cells compared to control, whereas the expression of MMP-2 has not been found increased.

5.2.3 Zymography assays demonstrating increased proteolytic activities of induced MMPs

Zymography is considered as a very sensitive, quantifiable and functional method. It was used in our study to determine the activities of MMPs by identification of their preferential substrates and molecular weights. Gelatin zymography was mainly performed to detect the gelatinases MMP-2 and MMP-9. Higher expression and activity of MMP-9 compared to control was seen after incubation with TMZ (500µM) for 5 days, whereas that of MMP-2 has not been evidently changed. Casein zymography helped to determine the activity of MMP-1. The expression level of MMP-1 in U87-MG cells is normally very low (qPCR data: CT value of MMP-1 is about 33 cycles), so that the anti-hMMP-1 antibody hardly detects MMP-1. In the separating gel of Casein zymography, a more intense 48 kDa active MMP-1 band has been identified in the concentrated supernatant sample of U87-MG cells treated with TMZ (500µM) for 5 days compared to control, which revealed increased activity of MMP-1 after TMZ treatment.

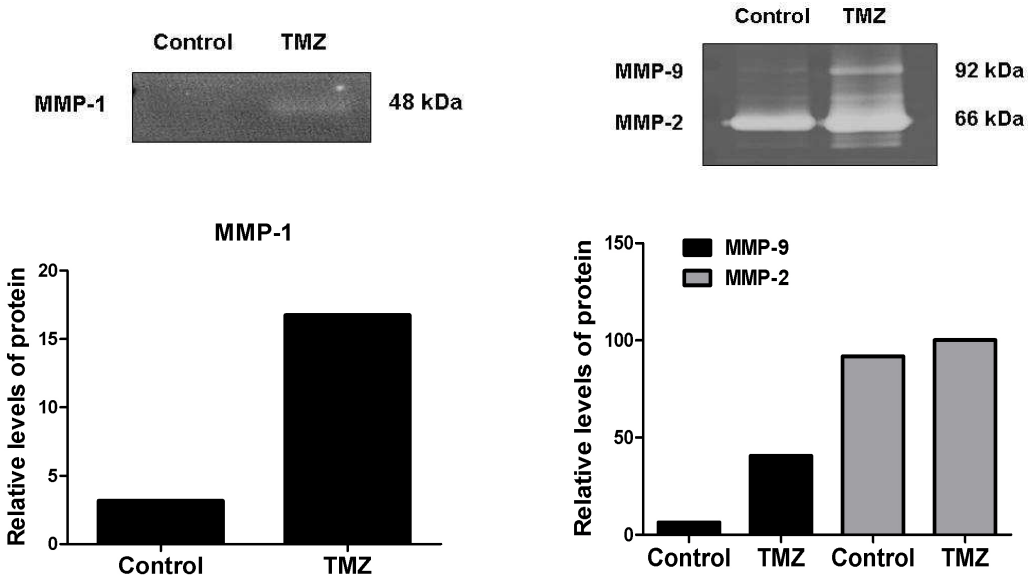

Figure 9. Detection of MMP activities in supernatants of U87-MG cells by Casein and Gelatin zymography assays

Protein concentrations were determined using BCA protein assay and equal amount of protein was added in each lane. Increased expression levels and activities of MMP-1 and MMP-9 were shown in samples of U87-MG cells treated with TMZ for 5 days.

5.2.4 Fluorescence activity assays for ADAM8 and MMP-1

To determine the increased expression of ADAM8 and MMP-1 at mRNA/protein level resulting in enhanced proteolytic activity, activity assays were performed using concentrated supernatants of U87-MG cells under different culture conditions. PEPDAB013 and PEPDAB008 (BioZyme Inc.) were employed to be the specific substrate for ADAM8 and MMP-1, respectively. Higher activities of ADAM8 and MMP-1 have been found in the cell supernatant after cells exposure to TMZ (500µM) for 5 days in comparison to control, which revealed that TMZ could not only induce the expression of ADAM8 and MMP-1, but enhance their proteolytic activities as well.

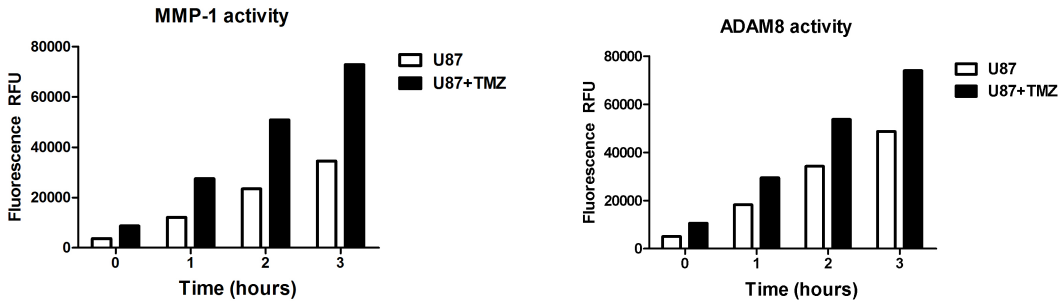

Figure 10. Activity assay determining the promoted proteolytic activities of ADAM8 and MMP-1

Fluorometric analysis of protein activities for MMP-1 and ADAM8 in U87-MG cells treated with TMZ for 5 days showed significant mRNA/protein inductions in comparison to control. Activity assays were performed using specific substrates for MMP-1 and ADAM8, respectively. Substrate cleavage was determined by the fluorometric method and the result demonstrated that TMZ treatment not only induced the expression of MMP-1 and ADAM8, but enhanced their proteolytic activities as well.

5.3 TMZ incubation enhanced invasiveness of U87-MG cells

The guiding concept concerning the roles of metalloproteases in glioma has been the metalloproteases-mediated degradation of extracellular matrix (ECM) resulting in tumour cells invasion and metastasis over the past decades. To demonstrate the functional role of up-regulated metalloproteases in U87-MG cells after TMZ

treatment, we performed Matrigel™ invasion assays. When U87-MG cells were incubated with TMZ (500µM) for 5 days, they displayed a significantly increased invasive ability through Matrigel™ determined by counting the number of cells that migrated to the bottom of the invasion chamber. The results demonstrated clearly that the induction of metalloproteases in U87-MG cells by TMZ caused increased invasiveness of the tumour cells, which could be an essential mechanism of chemoresistance of malignant glioma cells.

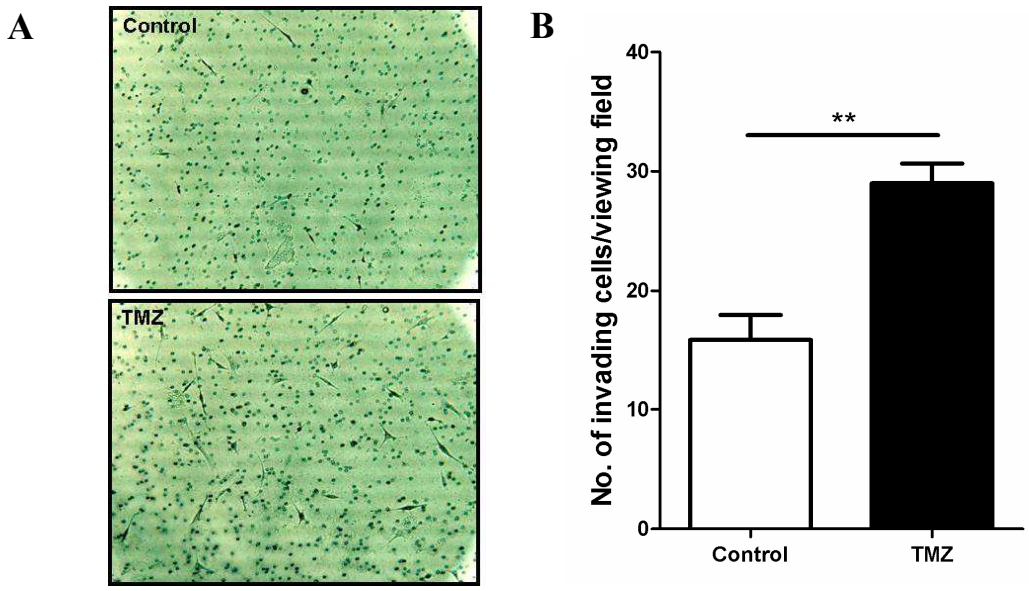

Figure 11. Invasion assay to examine the invasiveness of U87-MG cells before and after TMZ treatment

A) Cells attached to the bottom of the invasion chamber stained with Hematoxylin have been found under bright-field microscopy. **B)** Relative numbers of invaded U87-MG cells were counted in randomly selected viewing fields. After TMZ treatment, much more cells migrated through the Matrigel™ showing promoted invasive ability of U87-MG cells compared to control. Values are presented as mean, SEM, n=5. Significant differences, paired t-test: **p<0.01.

5.4 Effect of BB-94（Batimastat）on U87-MG cells treated with TMZ

5.4.1 BB-94 sensitizes U87-MG cells to TMZ using MTT assay

The data above demonstrated that the up-regulated expression levels and activities of some metalloproteases caused by TMZ could be involved in the mechanism of

chemoresistance of glioma cells, therefore, inhibition of these metalloproteases could result in promoted sensitivity of U87-MG cells to TMZ. To analyse that, we used the broad-spectrum MP inhibitor (BB-94), which is capable of inhibiting MMP-1 (IC_{50}=3nM), MMP-9 (IC_{50}=4nM), MMP-14 (IC_{50}=2.1nM) and ADAM8 (IC_{50}=51.3nM).

We performed MTT assays using BB-94 (0.02μM) to co-incubate U87-MG cells with TMZ (500μM). After incubation for 3 days, almost no difference in the percentage of viable cells has been found between two groups with and without BB-94. However, around 20% less viable U87-MG cells remained when treated for 5 days with BB-94 and TMZ compared to the group without BB-94 (55% versus 75%). A smaller difference was seen in U87-MG cells cultured for 7 days (25% versus 35%). Real-time PCR analysis showed that after TMZ treatment for 5 days, expression levels of ADAM8, MMP-1, MMP-9 and MMP-14 in U87-MG cells were found significantly increased. This MTT assay also revealed a notable difference in cell viability after incubation with TMZ for 5 days. These data suggest that the up-regulated metalloproteases result in enhanced proliferation of malignant glioma cells and inhibition of these proteases causes decreased proliferation and increased sensitivity of tumour cells to TMZ.

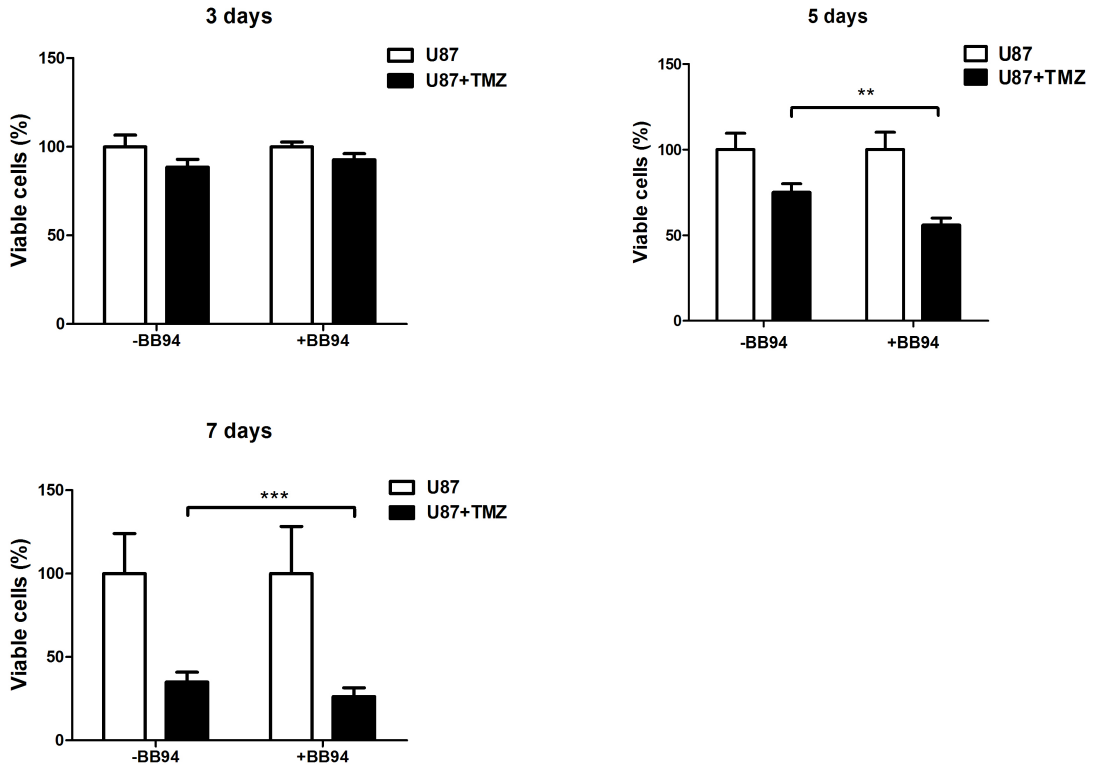

Figure 12. MTT assay to examine chemosensitivity of U87-MG cells caused by inhibition of metallproteases

BB-94 was used to inhibit the activities of metalloproteases. It was revealed that BB-94 in combination with TMZ led to less viable tumour cells remaining compared to cells treated with TMZ alone. Values are presented as mean, SEM, n=6. paired t-test: **p<0.01, ***p<0.001.

5.4.2 BB-94 offsets the promoted invasiveness of U87-MG cells by TMZ

Invasion assays were performed to determine the lowered invasive ability of U87-MG cells caused by BB-94 incubation. U87-MG cells alone don't show any invasive ability through Matrigel™, however, TMZ significantly increased invasiveness of U87-MG cells judged by the number of cells migrated to the bottom of invasion chambers in our study. Using BB-94 to inhibit the function of metalloproteases resulted in very low migration rate through Matrigel™. These results verified properly our hypothesis mentioned above, that is, the up-regulation of metalloproteases resulting in increased proliferation and invasiveness of malignant glioma cells is an important aspect involved in the chemoresistance of glioma cells, which could be

overcome by inhibition of these endogenous peptidases.

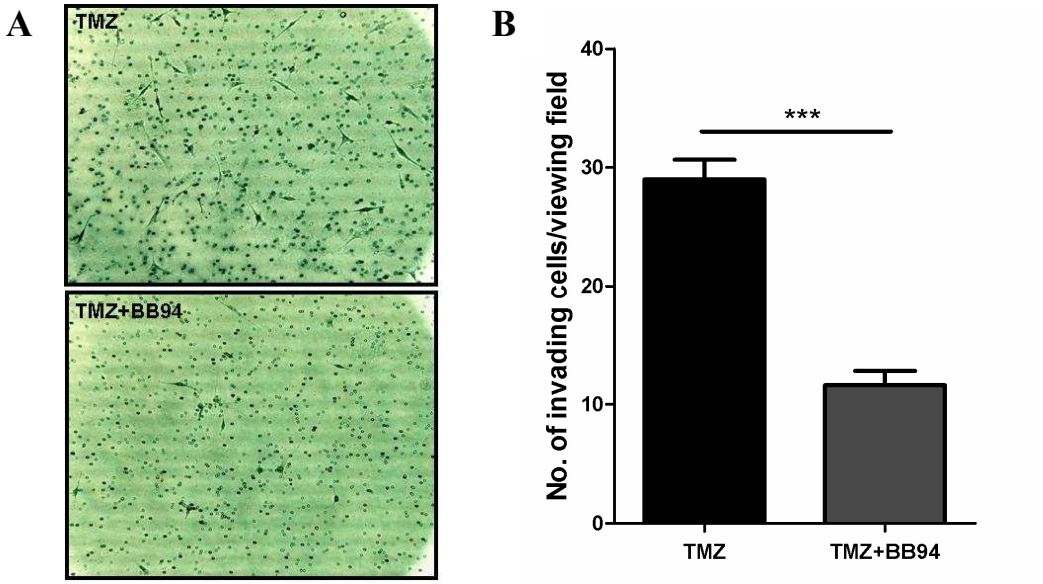

Figure 13. Inhibited invasiveness of U87-MG cells caused by BB-94
A) Invading cells attached to the bottom of invasion chambers under a light microscope. **B)** Relative numbers of invading cells were counted in randomly selected viewing fields. BB-94 lowered the invasive ability of U87-MG cells effectively. Values are presented as mean, SEM, n=6. Significant differences, paired t-test: ***p<0.001.

5.5 Effect of TMZ on intracellular ERK signalling

5.5.1 UO126 partly counteracts TMZ-induced MPs

Mitogen-activated protein kinases [MAPKs, also called extracellular signal-regulated kinases (ERKs)] constitute a signal cascade transduction pathway from cell surface to nucleus, which participates in a variety of cellular events, from embryogenesis and differentiation to cell proliferation and death. To determine that MAPKs pathway serves as an essential machinery underlying the induction of MPs by TMZ, UO126 was applied to inhibit ERK1/ERK2 pathway and helped find the potential effect of MAPKs signalling on the increased expression of MPs. We treated U87-MG cells with UO126 of 10μM in combination with TMZ (500μM) for 5days and harvested the cells. Western blot was used to determine the inhibitory effect of UO126 on activation of ERK1/ERK2 (Fig 14.). In comparison to control, cells treated with TMZ displayed

enhanced active ERK1/ERK2, whereas UO126 blocked this activity of TMZ. ß-Tubulin was used to provide a loading comparison. Real-time PCR has been performed to demonstrate the suppressive influence of UO126 on the expression of MPs at mRNA level (Fig 15.). The induced expression of ADAM8, MMP-1 and MMP-9 by TMZ has been significantly decreased by UO126 incubation and the lowered expression approached baseline expression of these MPs in U87-MG cells. However, UO126 has not counteracted the increased expression of MMP-14, and even slightly enhanced the induced expression of MMP-14 by TMZ.

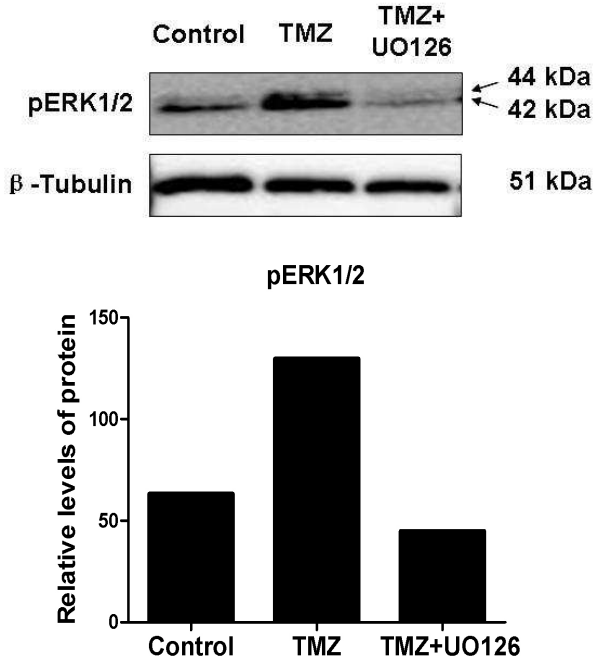

Figure 14. Western blot to analyse the effect of UO126 on the activation of pERK1/2. After incubation with TMZ for 5 days, expression of activated ERK1/2 in U87-MG cells was increased. However, UO126 reversed the induction of pERK by TMZ.

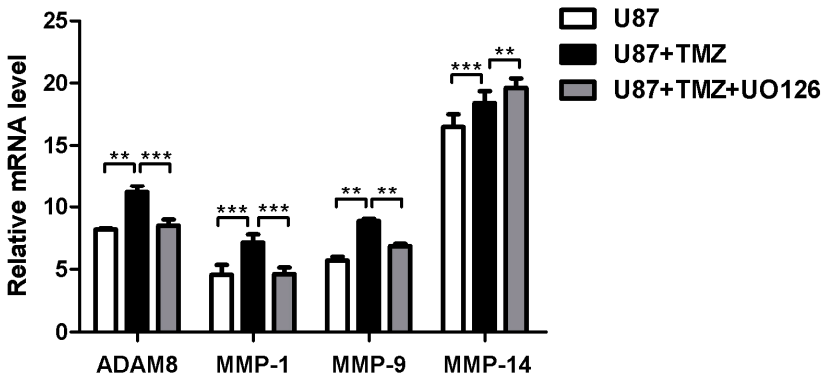

Figure 15. Real-time PCR to demonstrate influence of UO126 on the expression of induced MPs by TMZ

UO126 significantly decreased the up-regulated expression of ADAM8, MMP-1 and MMP-9 by TMZ. The promoted expression of MMP-14 was not offset by UO126, but slightly enhanced. Values are presented as mean, SEM, n=6. Significant differences, paired t-test: **p<0.01, ***p<0.001.

5.5.2 Effect of UO126 on the chemoresistance and invasiveness of U87-MG cells

Given that UO126 has been shown to reverse the increased expression of ADAM8, MMP-1 and MMP-9, we further performed MTT assays and invasion assays to detect its influence on the malignant phenotype of U87-MG cells. MTT assays demonstrated that UO126 caused rising sensitivity of U87-MG cells to TMZ. When compared to the group with TMZ treatment alone, about 5% less viable cells remained after incubation with UO126 and TMZ for 5 days (Fig 16.). Inhibition of MAPK pathway resulted in significantly decreased invasiveness of U87-MG cells (Fig 17.).

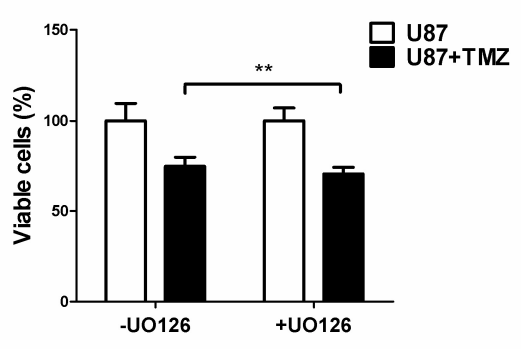

Figure 16. MTT assay to examine the effect of UO126 on the susceptibility of U87-MG cells to TMZ

In comparison to cells without UO126 treatment, UO126 incubation results in about 5% less viable cells remaining. Values are presented as mean, SEM, n=3. paired t-test: **p<0.01.

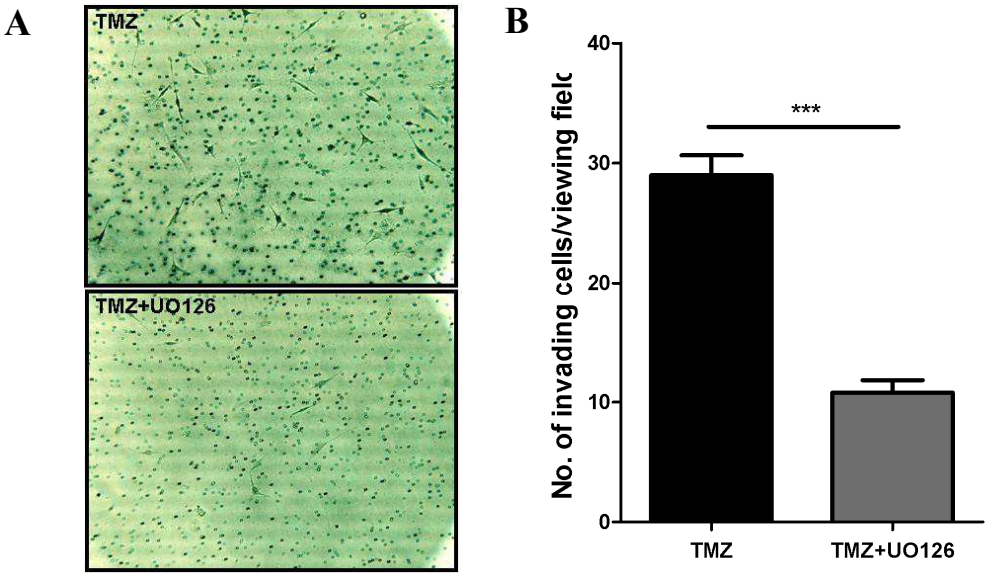

Figure 17. Invasion assay to survey effect of UO126 on the invasiveness of U87-MG cells

UO126 offset the increased invasiveness of U87-MG cells caused by TMZ. **A)** Images of invading cells attached to the lower side of invasion chambers. **B)** Relative numbers of invading cells were counted in randomly selected viewing fields. Values are presented as mean, SEM, n=6. Significant differences, paired t-test: ***p<0.001.

5.6 DAPT decreases the resistance of U87-MG cells towards TMZ

PS/γ-secretase combined with metalloproteases (MPs) are involved in a variety of trans-membrane proteins activation and signalling pathways. To investigate the involvement of γ-secretase in conferring chemoresistance, MTT assays were performed using DAPT (1μM) as an inhibitor of γ-secretase. U87-MG cells were

cultured for 5 days and the data showed that DAPT+TMZ caused about 6% less viable cells compared to treatment of TMZ alone (Fig 18.), which indicated that γ-secretase was also associated with the resistance of U87-MG cells towards TMZ.

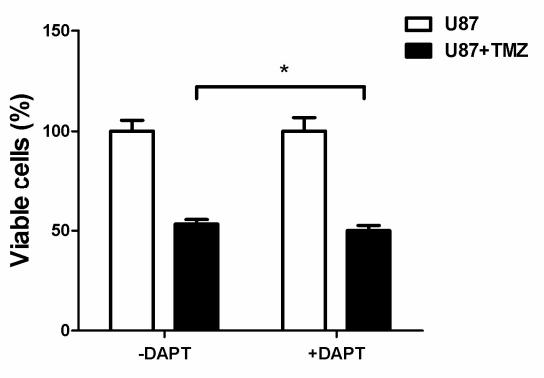

Figure 18. MTT assay to determine the role of γ-secretase in the resistance of U87-MG cells against TMZ

Incubation with DAPT, an inhibitor of PS/γ-secretase, caused 6% less viable cells remaining. Values are presented as mean, SEM, n=6. Significant differences, paired t-test: *p<0.05.

6 Discussion

Despite great advantages in treatment strategies developed over the past decades, the long-term survival rate of patients with glioblastoma remains very low and this tumour type is still a major challenge in the field of neurosurgery (Torres *et al.*, 2011). TMZ is a second-generation alkylating agent widely applied to treat malignant glioma (Villano *et al.*, 2009). Acquired resistance of glioma cells to TMZ limits its efficacy (Kitange *et al.*, 2009; Oliva *et al.*, 2011; Strik *et al.*, 2012; Ulasov *et al.*, 2011). This study was encouraged by recent observations that the cell adhesion L1 is up-regulated under irradiation conditions and interferes after MP-mediated proteolytic cleavage with the DNA Damage Response machinery (Cheng et al., 2011). Therefore, our study aims to explore a potential contribution of metalloproteases to chemoresistance of glioblastoma upon TMZ treatment.

A Disintegrin And Metalloprotease (ADAM) family and Matrix Metalloproteases (MMPs) are well known endogenous metalloproteases, which bind a Zn^{2+} ion in the active site and catalyze the hydrolysis of non-terminal peptide bonds (Creighton, 1993). They play important roles in various physiological processes and pathogenesis or progression of cancers. It is shown that these metalloproteases are increased in many types of tumours and contribute to almost all aspects of malignant phenotype of cancers. In glioma specimens, elevated expression of ADAM and MMPs are as well involved in the proliferation, migration, neovascularisation, invasion and radioresistance of tumour cells. We hypothesized therefore that increased expression of metalloproteases also contributes to chemoresistance of glioma cells to TMZ.

In our study, TMZ was found to exert its inhibitory effect on U87-MG cells in a dose- and time-dependent manner (Fig 6.). After relative short-term incubation (3 days) with TMZ in increasing concentrations, 500μM TMZ revealed a significant suppressive effect with only 75% viable cells remaining, whereas TMZ of ≤200μM showed a weak effect compared with control. However, after incubation with TMZ

for 5 or even 7 days, a remarkable decrease in surviving cells was detected even with TMZ at lower concentrations. The mechanism by which TMZ acts on the cells (DNA damage and induction of DDR) is expected to be a long-term effect. At present, two groups of alkylating agents are used to treat cancers (TMZ and so-called CNUs) (Beier *et al.*, 2011). Different from CNUs, TMZ needs a sufficient DNA repair system to be effective, and it is the tertiary lesions formed during faulty mismatch repair, not the primary lesions caused by TMZ that induces cell death of affected tumour cells (Beier *et al.*, 2011). Roos and co-workers also showed that TMZ-induced and dose-dependent apoptosis in glioma cells occurred at late time points starting after 72-96 hours (Roos *et al.*, 2007). In addition, it is demonstrated that even with long-term incubation with TMZ of high concentrations, about 30% U87-MG cells survive in our study, which indicates that in addition to causing apoptosis, TMZ has also a cytostatic effect on tumour cells. Hermisson and colleagues described a similar result that even high concentrations of TMZ (up to 1290µM) led to up to 40% surviving glioma cells (Hermisson *et al.*, 2006).

Sequentially, we surveyed changes in expression levels of metalloproteases caused by TMZ. As a long-term expression change, we observed up-regulation of MMP-1, -9, -14 and ADAM8 at mRNA, protein and activity level after U87-MG cells have been treated with TMZ (500µM) for 5 days. At mRNA level, expression levels of these MPs were 6-fold, 5-fold, 4-fold and 14-fold increased, respectively (Fig 7.). However, some other MPs, even MMP-2, ADAM10 and ADAM17 expressed in glioma in significantly high expression, were not detected to be induced by TMZ. Protein analyses including Western blot, zymography assays and activity assays were employed to demonstrate the elevated expression and proteolytic activities of the MPs caused by TMZ (Fig 8., 9., and 10.). MMP-1, which is not substantially expressed in normal brain tissue, has been found elevated in glioma cells (Hodgson *et al.*, 2009) and corresponds to tumour severity and survival time of patients (Stojic *et al.*, 2008). MMP-9 is one of the two most abundant MMPs found in gliomas (Hormigo *et al.*, 2006; Komatsu *et al.*, 2004; Nakada *et al.*, 2003) and involved in tumour biology

(Ezhilarasan *et al.*, 2009; Lakka *et al.*, 2002). MMP-14 has as well been demonstrated to be over-expressed in gliomas and contribute to the active migration and invasion of tumour into normal brain tissue (Nakada *et al.*, 2003; Van Meter *et al.*, 2004). Furthermore, ADAM8 is highly regulated in gliomas and its expression level is associated with invasiveness (Wildeboer *et al.*, 2006). All together, these induced MPs by TMZ play important roles in malignant behaviour of gliomas, which makes us consider that they could probably be involved in the chemoresistance.

BB-94, serving as a broad-spectrum inhibitor of metalloproteases, was used in our study to demonstrate inhibition of MPs resulting in increased sensitivity of glioma cells to TMZ therapy. BB-94 combined with TMZ led to increased apoptosis of U87-MG cells in comparison to another group without BB-94 incubation after we treated the cells for 5 days or 7days (Fig 12.), whereas for 3 days, no significant distinction was detected between the two groups. We therefore assume that it is the up-regulated MPs that contribute to the resistance when integrating this result with the data of qPCR and protein analyses, and inhibition of these MPs causes susceptibility of U87-MG cells to TMZ. When comparing the percentage of remaining viable cells between the two groups, around 20% difference was shown. However, Devy et al described that specific inhibition of MMP-14 activity yielded no less than 70% tumour growth inhibition in breast cancer xenograft mouse models in which MMP-14 was over-expressed (Devy *et al.*, 2009). Our understanding of the hydroxamate-based inhibitor BB-94 is still far from complete. It is not a selective inhibitor for a certain MP and it is reported that ADAM proteases are not inhibited specifically by BB-94 (Koller *et al.*, 2009). Therefore, BB-94 is not considered as an ideal agent to inhibit functions of MPs and specific inhibitors are promising and becoming the focus of cancer research (Zucker and Cao, 2009). Nevertheless, the promoted susceptibility of U87-MG cells to TMZ via inhibition of MPs by BB-94 implicates that induced expression of MPs contributes to the resistance of glioma cells to TMZ.

Invasion assays demonstrated that up-regulated expression of ADAM8, MMP-1,

MMP-9 and MMP-14 by TMZ causes promoted invasiveness of U87-MG cells, which was in turn suppressed by an inhibitor of MPs, BB-94 (Fig 11,13). As mentioned above, BB-94 does not work specifically on ADAMs, therefore increased MMPs caused by TMZ are considered as the main contributors to enhanced invasiveness. Inhibition of these MPs results in lowered invasive ability of U87-MG cells. Since TMZ is a standard therapy for glioma, the increased expression of MPs causes enhanced invasiveness as a response of glioma cells to TMZ leading to tumour cells invading surrounding normal brain tissues and is therefore a critical machinery underlying the chemoresistance and recurrent tumour formation. In agreement with this hypothesis, we found in a proteomic screening (in collaboration with Dr. Oliver Schilling, Freiburg University) comparing U87-MG cells (TMZ) with U87-MG cells (TMZ+BB-94) that BB-94 prevents shedding of CD44 from U87-MG cells treated with TMZ (data available on request). Since CD44 (hyaluronic acid receptor) is crucial for glioma migration and invasiveness, we conclude that the BB-94 effect on invasiveness could at least in part be due to lack of cleavage of CD44. Other potential candidates found in the proteomic screening are Met (HGF/SF, Scatter factor) and integrins $\beta 1$ and α_v, all of which could contribute to invasiveness of glioma cells.

In our study, we have employed other glioma cell lines, like for example U373, U251 and G28, to demonstrate the similar mechanism involved in the chemoresistance (data not shown). We found that using BB-94 of indicated concentration sensitized these glioma cells to TMZ as well, demonstrating that up-regulated MPs by TMZ are also involved in the resistance against TMZ in some other glioma cell lines.

The upregulation of MMP-2, MMP-9 and MMP-14 and their role in invasiveness has been described earlier (Gabelloni *et al.*, 2010; Trog *et al.*, 2006), however, no data in the use of BB-94 and on the mechanism of MMP regulation was provided in these reports.

It was also reported that in T98G glioblastoma multiforme (GBM) cell, epidermal growth factor (EGF) induces MMP-1 expression mainly via MAPK pathway (Anand

et al., 2011). Xie H and co-workers showed that up-regulated expression levels of MMP-7 and MMP-14 were associated with the increasing pathological grades in brain glioma tissues and UO126 significantly decreased the expression of MMP-7 and MMP-14 in U87-MG cells (Xie *et al.*, 2011). In our study, we also presume that TMZ induces the expression of ADAM8, MMP-1, MMP-9 and MMP-14 via MAPK pathway. We examined the effect of ERK1/ERK2 inhibitor on the expression of these induced MPs and sensitivity of U87-MG cells to TMZ. Our results demonstrated that TMZ induced expression of MPs in part via MAPK pathway, as UO126 decreased the TMZ-induced expression of ADAM8, MMP-1 and MMP-9 which contributed mainly to enhanced invasiveness of U87-MG cells. Furthermore, UO126 sensitized U87-MG cells to TMZ. However, UO126 exerted almost no effect or even a positive effect on the expression of MMP-14, which implicates that a more complex regulation of MMP-14 independent from ERK1/2 signalling after TMZ treatment.

Metalloproteases mainly contribute to shedding of ectodomains of a majority of membrane-bound proteins resulting in activation of these proteins, and subsequently, PS/γ-secretases are involved in releasing the remaining intracellular domains (ICDs) which play a role in a variety of cellular events. DAPT, an inhibitor of γ-secretase, was used to examine the impact of γ-secretase on the resistance of glioma cells to TMZ. Our data showed that DAPT promoted the sensitivity of U87-MG cells to TMZ treatment, which implicates that PS/γ-secretase has also an important role in the resistance of glioma cells to chemotherapy. Several trans-membrane proteins, for example Notch protein, EGF (epidermal growth factor) and NCAM-L1 (neural cell adhesion molecule L1) (Shah *et al.*, 2005), are likely to be the target proteins of the endogenous peptidases and confer the chemoresistance of glioma cells. Gilbert et al found that DAPT decreased chemoprotection and repopulation of TMZ-treated gliomas via Notch pathway and DAPT+TMZ treatment *in vivo* blocked significantly tumour progression (Gilbert *et al.*, 2010). Therefore, the results indicate that the induced MPs cause the resistance of glioma cells towards TMZ probably via cleaving some trans-membrane proteins involved in DNA damage response (DDR) or

promoting proliferation and invasiveness of glioma cells.

In conclusion, we find that metalloproteases play a crucial role in the resistance of glioma cells against TMZ which is a standard agent treating gliomas. It is revealed that TMZ incubation not only induces the expression of MMP-1, MMP-9, MMP-14 and ADAM8, but enhances their proteolytic activities as well. BB-94 serving as a broad-spectrum inhibitor of MPs in combination with TMZ leads to more apoptosis of U87-MG cells, that is, sensitizes U87-MG cells to TMZ. Furthermore, the promoted invasiveness of U87-MG cells by TMZ is also inhibited by BB-94. TMZ induces the expression of MPs partly via MAPK pathway. Inhibition of PS/γ-secretase also leads to increased susceptibility of glioma cells to TMZ.

Here we show that induction of MPs by TMZ is an important aspect that contributes to the chemoresistance of glioma cells by cleaving some membrane-anchored proteins participating in DDR or increasing proliferation and invasiveness of glioma cells, which in future therapy regimens could be overcome by the specific inhibition of MPs to prevent formation of recurrent glioma.

7. Summary

7.1 Summary

Glioblastoma multiforme (GBM) is the most malignant type of brain tumours bearing a grim prognosis with a median survival of 14.8 months. Despite therapeutic advances over the past decades, GBM treatment remains ineffective with temozolomide (TMZ) as a standard chemotherapeutic agent. Even using TMZ combined with surgical resection and radiotherapy, the 2-year survival for patients of high-grade gliomas remains very low. Understanding potential molecular mechanisms contributing to the resistance of glioma cells to TMZ is important for optimizing the existing and developing novel therapeutic strategies.

Metalloproteases (MPs) have been found to be increased in GBM and are associated with the malignant phenotype. In our study, we have systematically examined the changes in the expression of MPs in U87-MG cells after TMZ treatment and found that treatment of U87-MG glioblastoma cells with TMZ for at least 5 days induces expression of metalloproteases ADAM8, MMP-1, MMP-9 and MMP-14 in surviving U87-MG cells.

To analyse the function of these MPs, a broad-spectrum metalloprotease inhibitor batimastat (BB-94) was applied to U87-MG cells in conjunction with TMZ. BB-94 causes increased susceptibility of U87-MG cells to TMZ induced cell death and reduces the invasive potency of U87-MG cells, indicating that the induction of MPs by TMZ contributes to chemoresistance and recurrence of glioblastoma. As a potential mechanism of MP induction by TMZ we found that ERK1/2 phosphorylation in U87-MG cells is enhanced after TMZ treatment. Application of UO126, a specific ERK1/2 inhibitor, abrogates TMZ-induced expression of ADAM8, MMP-1 and MMP-9, but not of MMP-14, implicating that there are alternative pathways for the induction of MMP-14 by TMZ.

PS/γ-secretase combined with MPs participates in trans-membrane processing of numerous proteins thereby regulating various intracellular pathways. Inhibition of γ-secretase using DAPT had a similar effect as BB-94 on sensitization of U87-MG cells to TMZ, suggesting that γ-secretase could function as an adjuvant peptidase to MPs to be involved in the chemoresistance of glioblastoma cells.

Taken together, we conclude that the induction of particular MPs MMP-1, MMP-9, MMP-14, and ADAM8 by TMZ causes chemoresistance of glioblastoma cells, and these MPs confer chemoresistance via cleaving membrane-bound proteins, for example CD44, Met or integrins ß1 and/or α_v, which might be involved in DNA damage response (DDR) or in proliferation and invasiveness of tumour cells. Therefore, TMZ treatment combined with MP inhibitor appears as a feasible therapy option to optimize TMZ therapies and to prevent recurrent glioma formation.

7.2 Zusammenfassung

Glioblastoma Multiforme (GBM) ist der aggressivste hirneigene Tumor mit einer schlechten Überlebensprognose. Trotz Weiterentwicklung der multimodalen Behandlungsansätze, bestehend aus Tumorresektion und Radio- sowie Chemotherapie ist die Therapie des GBM bei einem durchschnittlichen Überleben von 2 Jahren nur bedingt erfolgreich. Als Standardchemotherapie kommt heutzutage das alkylierende Chemotherapeutikum Temozolomid (TMZ, Temodal®) zum Einsatz. Ein Grund für die ineffiziente Wirksamkeit von Chemo-und Radiotherapie ist die Ausbildung von Resistenzen in den Tumorzellen. Das Verständnis der Resistenzbildung auf molekularer Ebene ist daher sehr wichtig und könnte neue Ansätze zur Optimierung der Chemo- und Radiotherapie liefern.

Metalloproteasen (MPs) sind in GBMs erhöht exprimiert und stets mit einer schlechten Prognose verbunden. In dieser Studie haben wir die Expression von MPs nach TMZ-Behandlung in U87-MG Glioblastom-Zellen systematisch untersucht. Eine TMZ-Behandlung von 5 Tagen induziert in U87-MG Zellen eine erhöhte Expression von ADAM8, MMP-1, MMP-9 and MMP-14 in den überlebenden Zellen.

Um die Funktion der induzierten MPs zu untersuchen, wurde der Breitband MP-Inhibitor Batimastat (BB-94) in U87-MG Zellen in Verbindung mit TMZ eingesetzt. BB-94 führt zu einer erhöhten Sensitivität von U87-MG Zellen gegenüber dem TMZ-induzierten Zelltod und verringert das durch TMZ induzierte invasive Potenzial von U87-MG Zellen. Daraus folgt, dass die durch TMZ induzierten MPs sowohl zu einer Chemoresistenz als auch zu einer Rezidiv-Bildung beitragen können. Als potenzieller Signalweg der TMZ-vermittelten MP-Induktion in U87-MG Zellen wurde eine Zunahme der ERK1/2 Phosphorylierung unter TMZ-Behandlung nachgewiesen. Die Verwendung des ERK1/2 Inhibitors UO126 führte zu einer Abnahme der TMZ-induzierten Expression von ADAM8, MMP-1 and MMP-9, aber nicht von MMP-14, was darauf hindeutet, dass es alternative Signalwege bei der

Induktion von MMP-14 gibt.

PS/γ-Sekretase kombiniert mit MPs führt zu einer Prozessierung von Transmembran-Proteinen, durch die zahlreiche intrazelluläre Signalwege gesteuert werden. Die Inhibition von γ-Sekretase durch DAPT hat in unseren Analysen einen vergleichbaren Effekt wie BB-94 auf die Sensitivierung von U87-MG Zellen durch TMZ, was darauf schließen lässt, dass γ-Sekretase zusammen mit MPs zu einer Chemoresistenz von Glioblastom-Zellen führen kann.

Zusammenfassend lässt sich sagen, dass die Induktion der Metalloproteasen MMP-1, MMP-9, MMP-14, and ADAM8 durch TMZ eine Chemoresistenz von Glioblastom-Zellen vermitteln kann und dass diese Metalloproteasen durch die Spaltung von Membranproteinen wie z.B. CD44, Met oder Integrinen an der „DNA Damage Response“ beteiligt sind oder die Proliferation und die Invasivität von Glioblastom-Zellen fördern können. Deshalb wäre eine kombinierte Therapie von TMZ mit Metalloprotease-Inhibitoren eine mögliche Therapie-Option, um die Effizienz der Standard-TMZ-Therapie zu verbessern und die Rezidivneigung zu verhindern.

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9. Appendix

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